Supporting information

Sulfamethoxazole and isoproturon degradation and detoxification by a laccase-mediator system: Influence of treatment conditions and mechanistic aspects

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1 Structures of the selected micropollutants and mediators

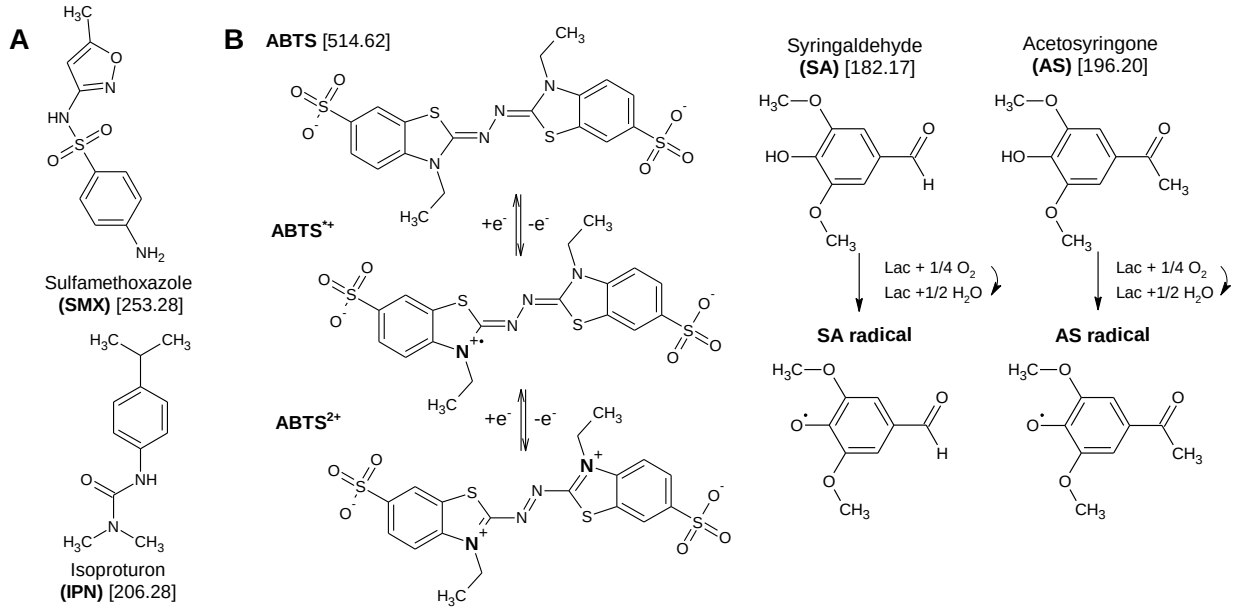

Fig. S 1 Selected micropollutants (A) and mediators (B). In (B), ABTS oxidation by laccase to its stable radical cation $ABTS^{\bullet+}$ and eventually to the di-cation $ABTS^{2+}$ (according to Fabbrini et al. [1]); and oxidation of the mediators syringaldehyde and acetosyringone to their respective unstable reactive phenoxy radicals (according to Martorana et al. [2]). In [], exact molar mass of the compounds (in g mol^{-1}).

2 Oxidation of IPN and SMX with various mediators

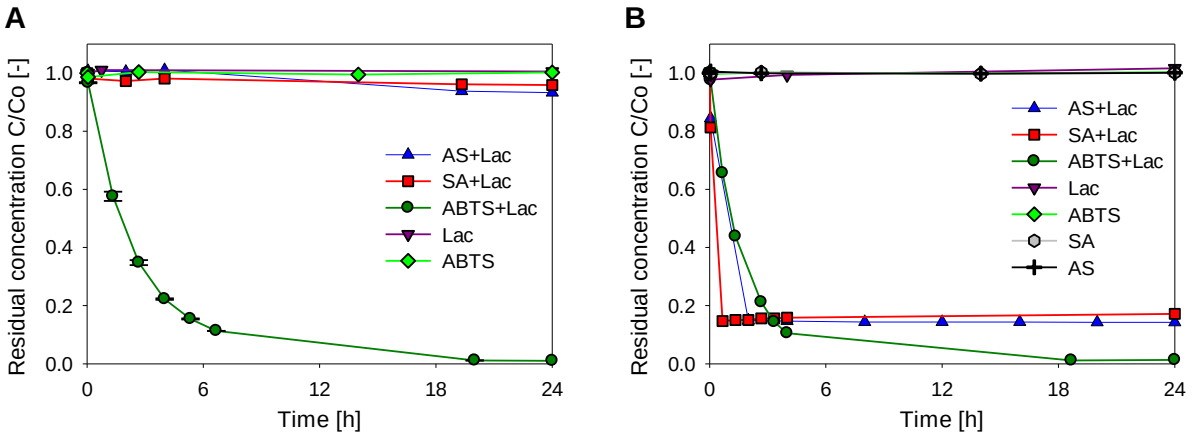

Fig. S 2 Relative residual concentrations of (A) isoproturon (100 µM) at pH 5 in presence of the 3 mediators at 500 µM and 630 U l^{-1} laccase activity, and **(B) sulfamethoxazole** (80-100 µM) at pH 6 in presence of the 3 mediators at 100 µM and 320 U l^{-1} laccase activity. Controls with only mediators (at 500 µM) and micropollutants (without laccase), as well as with micropollutants and laccase (without mediators) are also presented. Lac: laccase, AS: acetosyringone, SA: syringaldehyde, ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid).

3 Optimal pH for micropollutant abatement

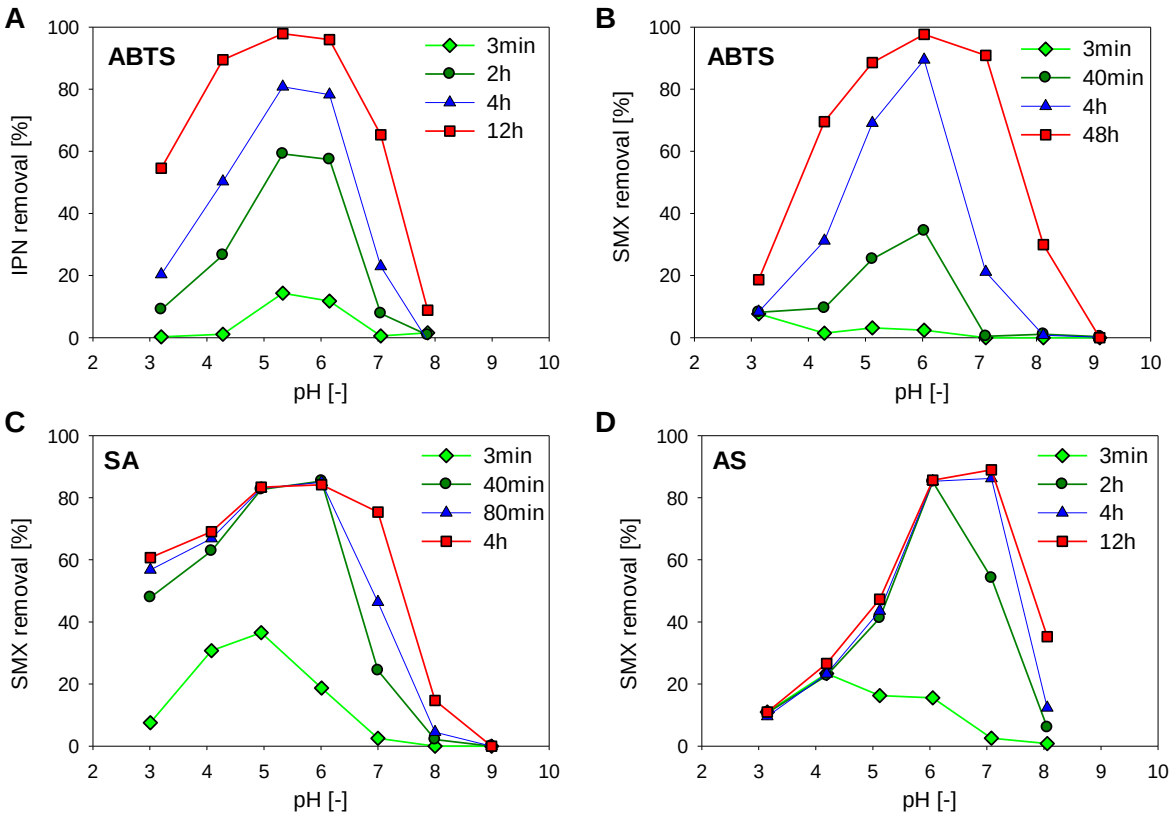

Fig. S 3 Influence of pH on the abatement of (A) isoproturon (IPN, 100 µM) with ABTS (500 µM, 560 U l^{-1} laccase), **(B)** sulfamethoxazole (SMX, 80 µM) with ABTS (100 µM, 315 U l^{-1} laccase), **(C)** SMX (80 µM) with syringaldehyde (100 µM, 320 U l^{-1} laccase), and **(D)** SMX (100 µM) with acetosyringone (200 µM, 560 U l^{-1} laccase). Results for different reaction times.

4 Correlation between mediator consumption and sulfamethoxazole removal

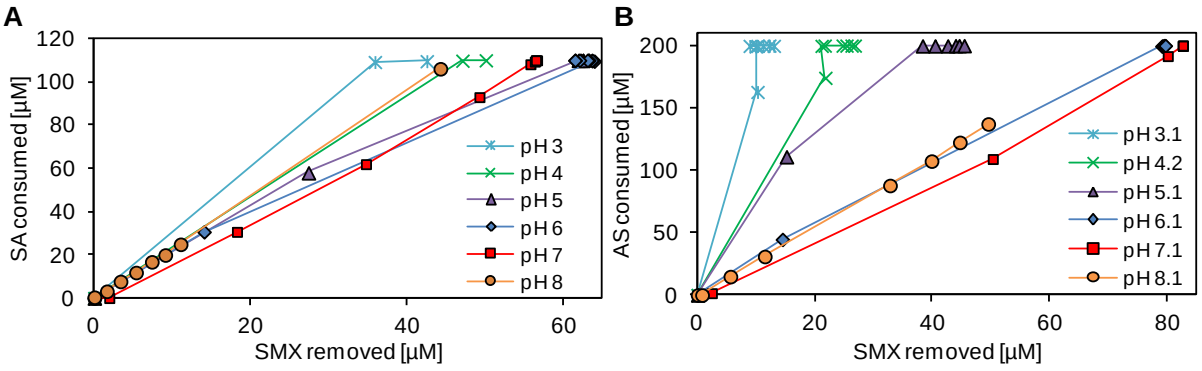

Fig. S 4 Correlation between sulfamethoxazole (SMX) removal and the mediator consumption at various pH values. **(A)** With syringaldehyde (SA) (initial concentrations of 75 and 110 µM for SMX and SA, respectively). **(B)** With acetosyringone (AS) (initial concentrations of 93 and 201 µM for SMX and AS, respectively).

5 Influence of enzyme, mediator and micropollutant concentrations on the rate of their abatement

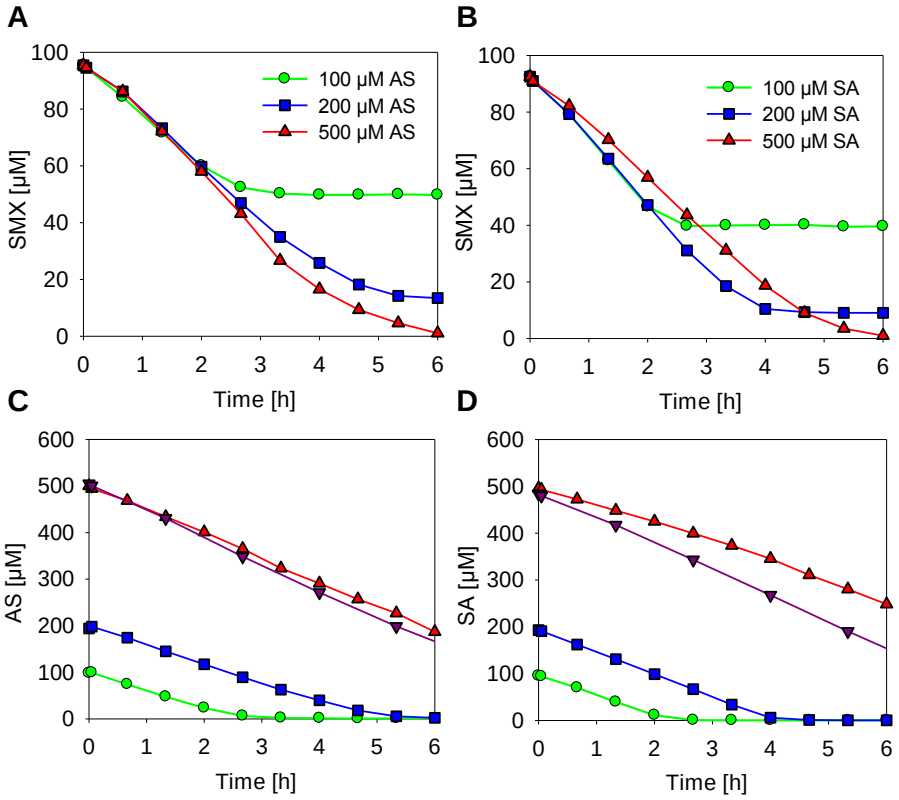

Fig. S 5 Influence of mediator concentrations on sulfamethoxazole oxidation (SMX, 91-95 µM) at pH 7. Residual SMX concentration with **(A)** acetosyringone (AS) at 100 (●), 200 (■) or 500 (▲) µM (520 U l^{-1} laccase); **(B)** syringaldehyde (SA) at 100 (●), 200 (■) or 500 (▲) µM (455 U l^{-1} laccase). Residual concentrations of **(C)** AS and **(D)** SA at initial concentrations of 100 (●), 200 (■) or 500 (▲) µM with SMX (91-95 µM), or 500 µM without SMX(▼) (same experiments as in A and B, respectively).

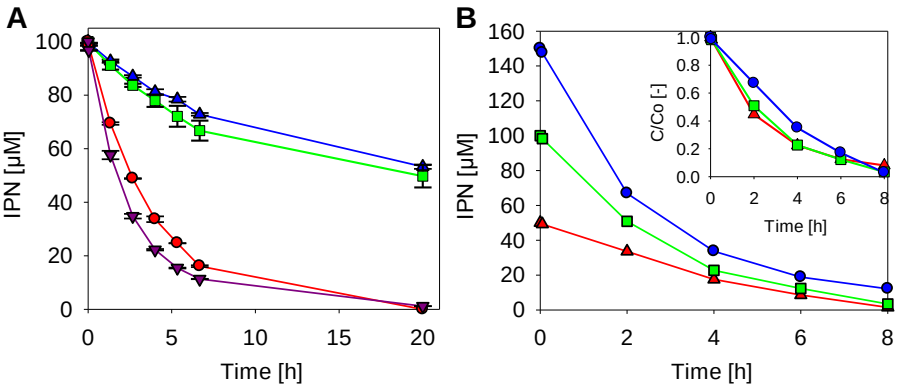

Fig. S 6 Influence of enzyme and mediator concentrations on isoproturon (IPN, 100 µM) oxidation at pH 5. (A) 100 µM ABTS and 120 U l^{-1} laccase (▲), 100 µM ABTS and 600 U l^{-1} laccase (■), 500 µM ABTS and 120 U l^{-1} laccase (●), and 500 µM ABTS and 600 U l^{-1} laccase (▼). **(B)** 560 U l^{-1} laccase and different concentrations of ABTS and IPN, but with a ratio of five (ABTS:IPN): 250:50 µM (▲), 500:100 µM (■) and 750:150 µM (●). Insert: relative concentration. Error bars: range of values of duplicates.

6 Dissolved oxygen consumption during laccase-mediator reactions

Dissolved oxygen consumption experiments were conducted in a closed (airtight) cell containing an oxygen probe and 3 ml of reactive solution. The cell was closed just after addition of laccase in an air-oxygen saturated solution, without any headspace.

Results: For AS and SA (Fig. S 7 A), SMX was completely removed in 6 h while oxygen was still above 60% saturation (no oxygen limitation). Approximately 0.8 and 1 mole of oxygen were consumed per mol of SMX oxidized, with SA and AS, respectively. Oxygen consumption stopped once SA was oxidized while, with AS, almost complete oxygen depletion was observed, possibly due to further oxidation of the transformation products. With ABTS (Fig. S 7 B), SMX was completely removed in about 3 h (still 60% oxygen saturation) and IPN in about 18 h (20% oxygen saturation), thus oxygen should not limit the reaction. About 1 and 2 moles of oxygen were thus consumed per mole of SMX and IPN oxidized, respectively. Complete oxygen depletion was observed during the reaction with ABTS, this occurring a long time after complete oxidation of ABTS to its radical cation. This suggests that the ABTS radical cation was further slowly oxidized to transformation products. Although the setup was not designed to calculate precisely the stoichiometry of the reaction (oxygen diffusion from the air was possible before closing the cell), it was observed that about 0.25 mole of oxygen was consumed (during the first period of the reaction) per mole of mediator (SA, AS and ABTS) oxidized, suggesting a one electron transfer from each mediator molecule.

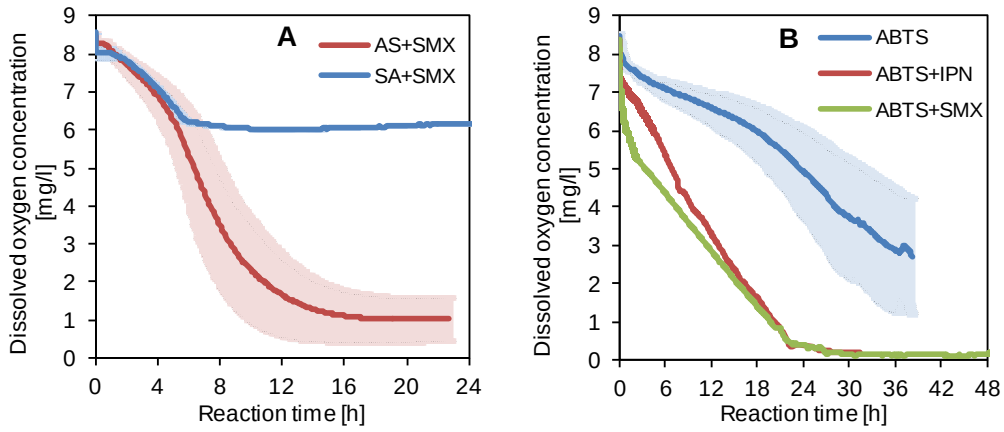

Fig. S 7 Dissolved oxygen consumption during laccase-mediated oxidation of sulfamethoxazole (SMX) and isoproturon (IPN). (A) Oxidation of SMX (100 µM) in presence of acetosyringone (AS) or syringaldehyde (SA) (500 µM) and 300 U l^{-1} laccase, at pH 7. **(B)** Oxidation of IPN or SMX (100 µM) in presence of ABTS (450 µM) and 300 U l^{-1} laccase, at pH 5, or under the same conditions but without pollutants (ABTS alone). Average and values (interval) of duplicates.

7 Role of ABTS radical cation in the oxidation

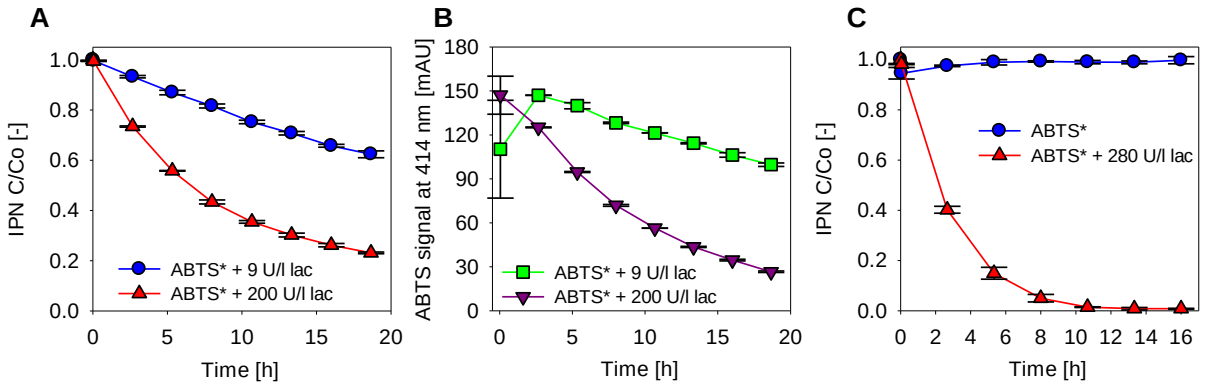

Fig. S 8 Oxidation of isoproturon (IPN, 100 µM) with oxidized ABTS (radical cation ABTS*) at pH 5, with or without laccase. (A) Residual IPN concentrations in presence of 205 µM ABTS$^{\bullet+}$ (500 µM ABTS oxidized by laccase and ultrafiltered to remove the enzyme): (●) ultrafiltered solution (9 U l^{-1} residual laccase activity), (▲) ultrafiltered solution with addition of 200 U l^{-1} laccase. **(B)** ABTS$^{\bullet+}$ concentration (UV-Vis signal at 414 nm) for the experiment described in A; (■) with ultrafiltered solution, (▼) with ultrafiltered solution and 200 U l^{-1} laccase. **(C)** Residual IPN concentrations with (●) 540 µM ABTS$^{\bullet+}$ (oxidized chemically with HOCl), or (▲) 540 µM ABTS$^{\bullet+}$ and 280 U l^{-1} laccase. Error bars: range of values of duplicates.

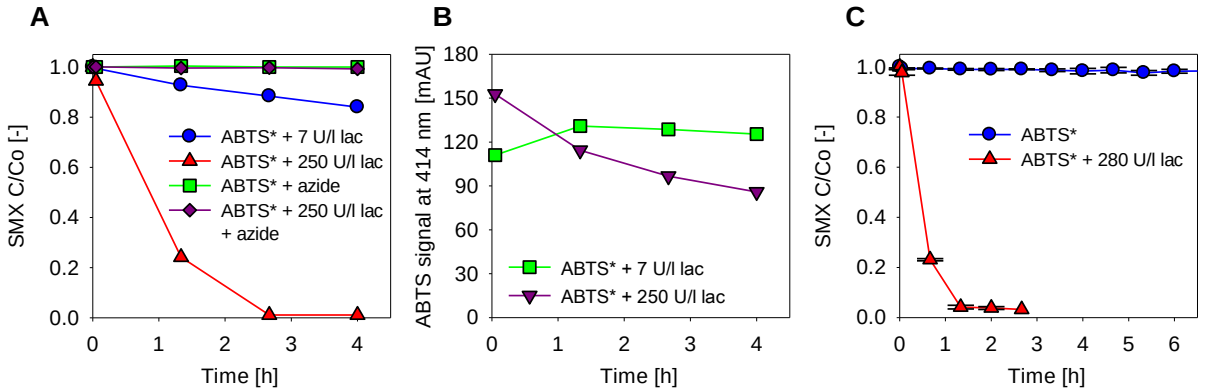

Fig. S 9 Oxidation of sulfamethoxazole (SMX, 100 µM) with oxidized ABTS (radical cation ABTS*), with or without laccase. (A) Residual SMX concentrations at pH 6 in presence of 160 µM ABTS$^{\bullet+}$ (500 µM ABTS oxidized by laccase and ultrafiltered to remove the enzyme): (●) ultrafiltered solution (7 U l^{-1} residual laccase activity), (▲) ultrafiltered solution with addition of 250 U l^{-1} laccase, (■) controls with ABTS$^{\bullet+}$ and sodium azide (10 mM), (◊) control with ABTS$^{\bullet+}$, laccase (250 U l^{-1}) and sodium azide (10 mM). **(B)** ABTS$^{\bullet+}$ concentration (UV-Vis signal at 414 nm) for the experiment described in A; (■) with ultrafiltered solution, (▼) with ultrafiltered solution and 250 U l^{-1} laccase. **(C)** Residual SMX concentrations at pH 5 with (●) 540 µM ABTS$^{\bullet+}$ (oxidized chemically with HOCl), or (▲) 540 µM ABTS$^{\bullet+}$ and 280 U l^{-1} laccase. Error bars in C: range of values of duplicates.

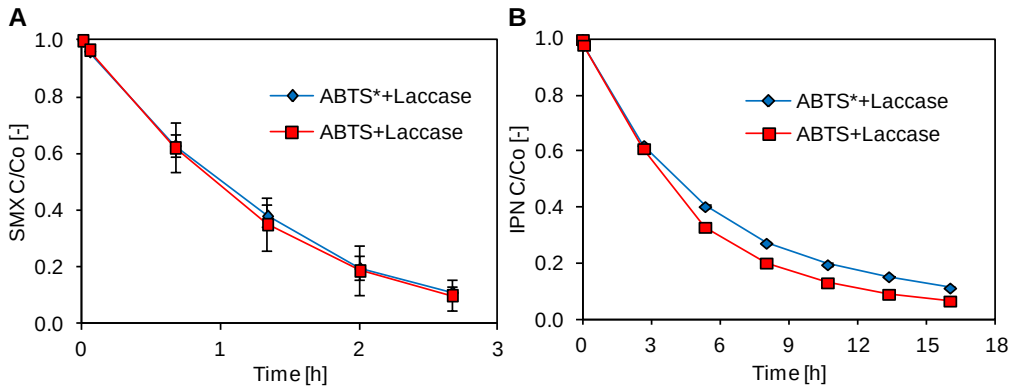

Fig. S 10 Oxidation of **(A)** sulfamethoxazole (SMX, 100 µM), and **(B)** isoproturon (IPN, 100 µM) with laccase (260 U l^{-1}) and either ABTS (■, 450 µM), or ABTS radical cation (◊, $ABTS^{\bullet+}$, 450 µM), at pH 5. $ABTS^{\bullet+}$ was produced before the reaction by chemical oxidation (at a stoichiometric ratio) of 450 µM ABTS with HOCl. Average and values (error bars) of duplicates.

8 Characteristics of parent compounds and transformation products

Table S 1 Characteristics of the parent compounds and the detected transformation products (TPs)

ID	Retention time [min]	Molar mass [g/mol]	Type of products[a]	Structures proposition[b]	Log K_{ow} of proposed structures[c] [-]	Maxima UV/Vis absorbance[d] [nm]
Parent compounds						
(AS)	7.11	196	Acetosyringone	(AS)	1.23	**218, 302**
(SA)	6.6	182	Syringaldehyde	(SA)	0.86	**218, 308**
(ABTS)	6.44 to 7.69	514	ABTS	(ABTS)	1.99	**224, 344**, oxidized: **414, 648**
(SMX)	6.19	253	Sulfamethoxazole	(SMX)	0.43	**200, 268**
(IPN)	10.62	206	Isoproturon	(IPN)	2.32	**200, 242**
Transformation products AS+SMX						
(1)	4.58	180	AS TP	-		**208, 390-400**
(2)	5.05	168	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)	0.28	200, **290**
(3)	5.85	182	AS TP	(V)	-0.36	
(4)	7.91	248	-	-		
(5)	8.11	447	*Coupling AS-SMX*	(III)	3.23	
(6)	8.77	332	Dimeric AS TP	(I)	0.04/1.97	**200, 260-270**, 340-350, 500
(7)	9.1	415	*Coupling AS-SMX*	-		
(8)	9.37	403	Coupling SMX-DMBQ	(8)	2.28	**200**, **314**, 405
(9)	9.56	417	*Coupling AS-SMX*	(IV)	3.14	**200, 306-310**
(10*)	10.06	445	*Coupling AS-SMX*	-		200, 218
Transformation products SA+SMX						
(2)	5.05	168	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)	0.28	200, **290**
(11)	6.93	281	-	-		**200, 262**, 502
(12)	8.38	318	Dimeric SA TP	(II)	-0.37/1.42	**200, 280**, 360, 560
(8)	9.37	403	Coupling SMX-DMBQ	(8)	2.28	**200**, **314**, 405
(13)	9.66	348	-	-		
Transformation products ABTS+SMX						
(14*)	2.01	258	ABTS TP	(14)	0.08	**200, 258**, 286, 294
(15*)	2.48	98	*SMX fragment*	(VI)	-0.74	**200, 265**
(16)	8.26	238	*SMX fragment*	(VII)	1.91	**200**, 220, 304, **410-420**
Transformation products ABTS+IPN						
(17*)	1.69	273	ABTS TP	(VIII)	0.4	**218, 258**, 284, 292
(14*)	1.99	258	ABTS TP	(14)	0.08	**200, 258**, 286, 294
(18*)	4.72	546	ABTS TP	-		200, **222, 264**, **292**, 300
(19)	7.23	222	*Hydroxy-isoproturon*	(IX)	1.85	
(20)	8.56	445	*Coupling IPN+fragment ABTS*	-		
(21)	8.9	447	*Coupling IPN+fragment ABTS*	-		
(22)	11.61	232	-	-		

[a] Transformation products may come from the mediator degradation by laccase (also observed without pollutant), or by reaction with the pollutant. *In italics*: suggestion based on the mass of the by-product. (-): no suggestion

[b] Refers to the structures proposed in Fig. S 11, based on the mass and the relation log Kow/retention time of the proposed molecule. (-): no suggestion

[c] Calculated for unionisated species with the software ACD/ChemSketch

[d] Determined by HPLC-DAD. In bold: main maxima. Transformation products with (*): correspondence between the compounds detected by UPLC-MS and HPLC-DAD not certain

9 Structural propositions for the transformation products

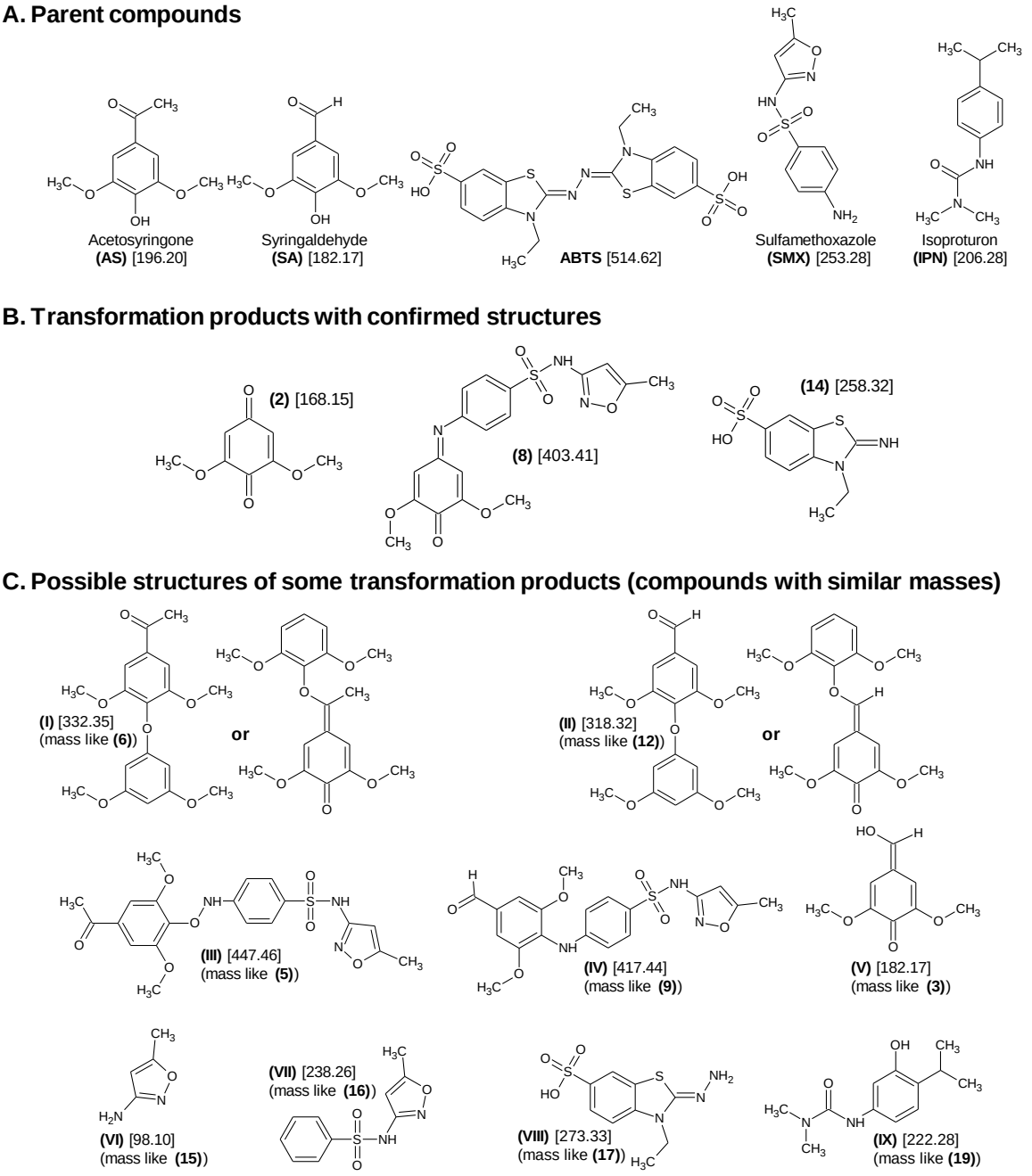

Fig. S 11 Structures and molar mass of (A) the parent compounds, (B) the transformation products with confirmed structures (by other related studies) and (C) compounds (numbered with roman numbers) with similar masses and properties (polarity) as some transformation products (Arabic numbers refer to the ID of the transformation products, cf. Table S 1 or Fig. 3, main manuscript) (hypothetical structures).

10 Kinetics of the formation of transformation products during laccase-mediated reactions

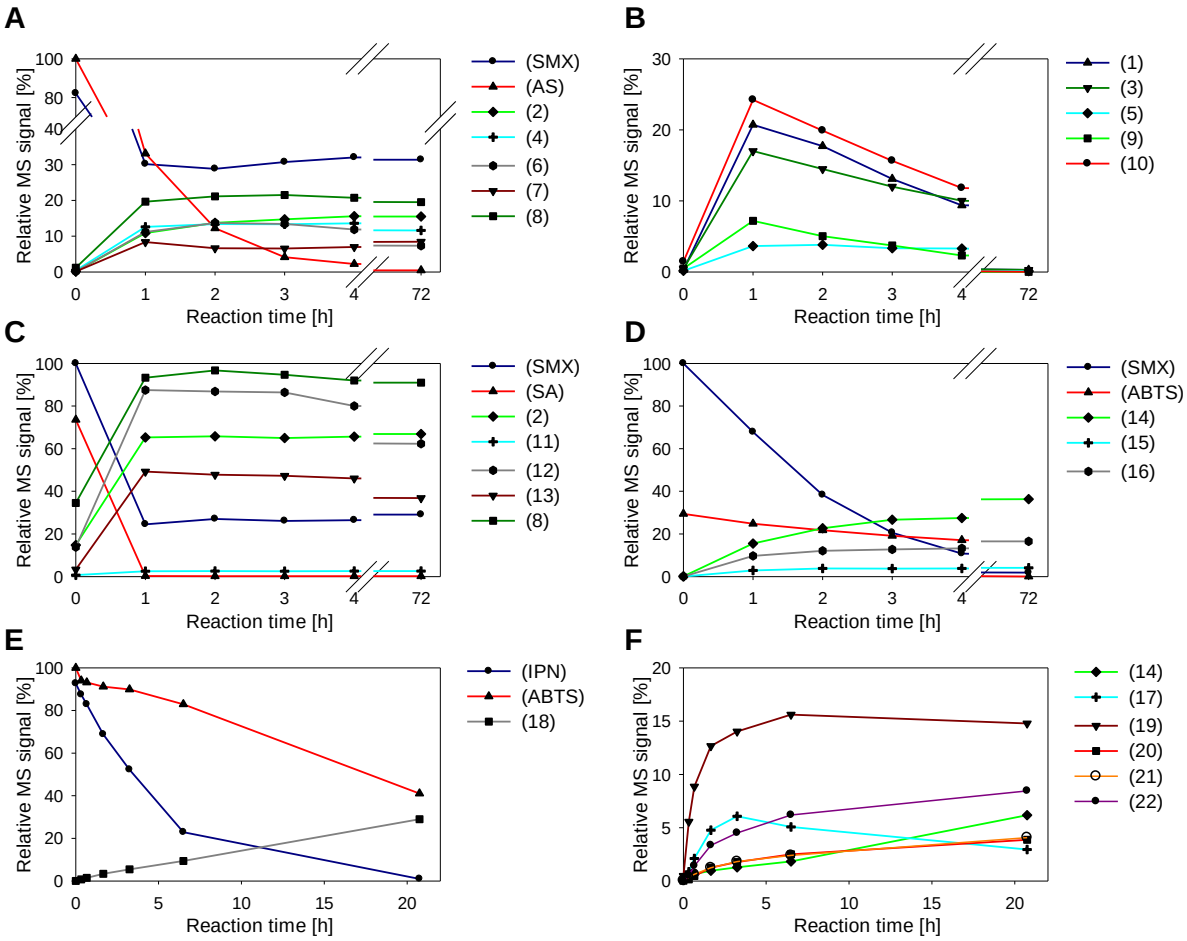

Fig. S 12 Kinetics of sulfamethoxazole (SMX) and isoproturon (IPN) oxidation and transformation products formation by laccase-mediator systems. (A and B) SMX in presence of acetosyringone (AS), (C) SMX in presence of syringaldehyde (SA), (D) SMX in presence of ABTS and (E and F) IPN in presence of ABTS. Numbers in brackets: ID of the transformation products, corresponding to Fig. 3 (main manuscript).

11 HPLC-DAD chromatograms

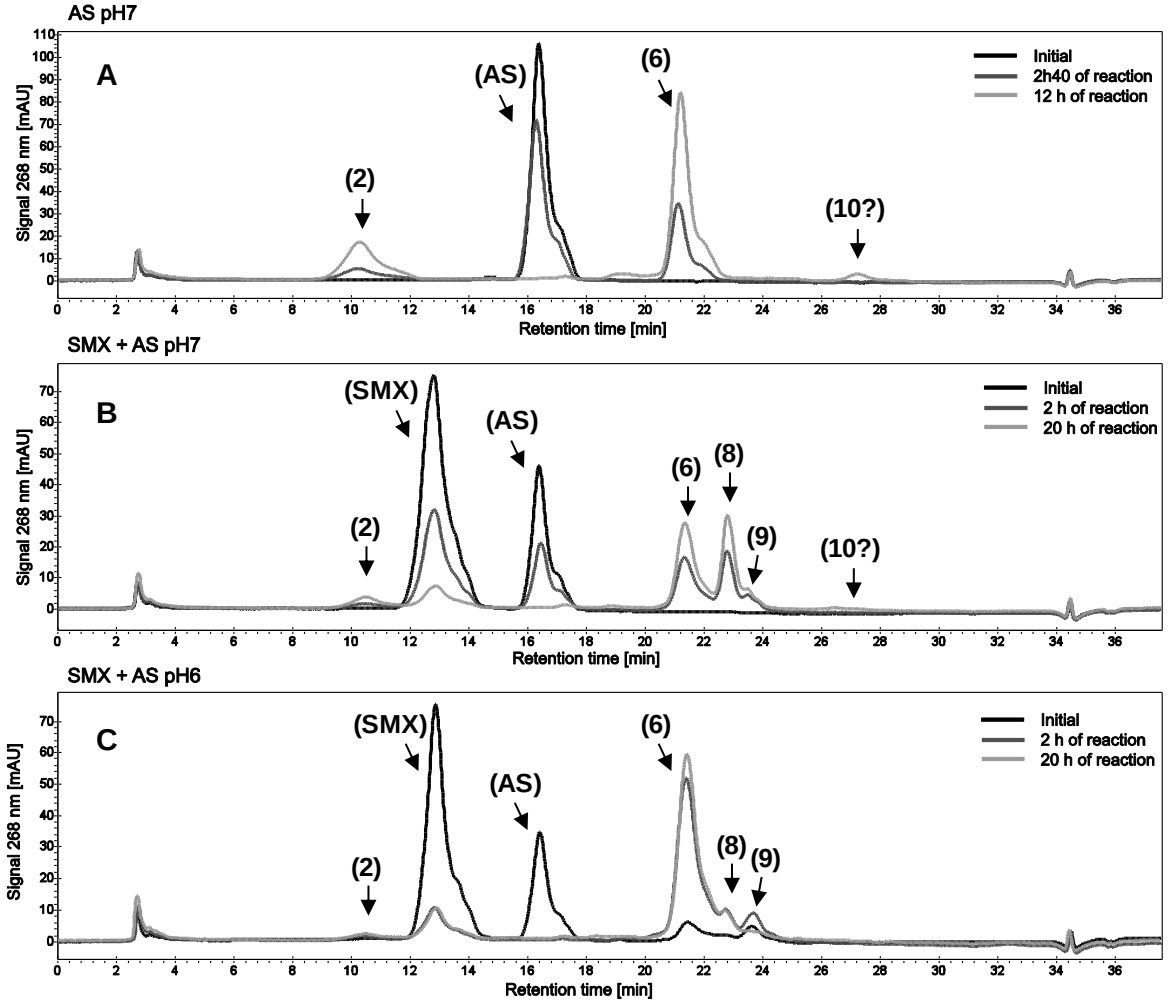

Fig. S 13 HPLC-DAD (268 nm) chromatograms during the oxidation of acetosyringone (AS) by laccase with or without sulfamethoxazole (SMX). (A) Oxidation of AS (500 µM) by laccase (450 U l^{-1}) at pH 7, without SMX. (B) Oxidation of AS (200 µM) by laccase (560 U l^{-1}) at pH 7, in presence of SMX (100 µM). (C) Similar conditions as in (B) but at pH 6. Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 3, main manuscript).

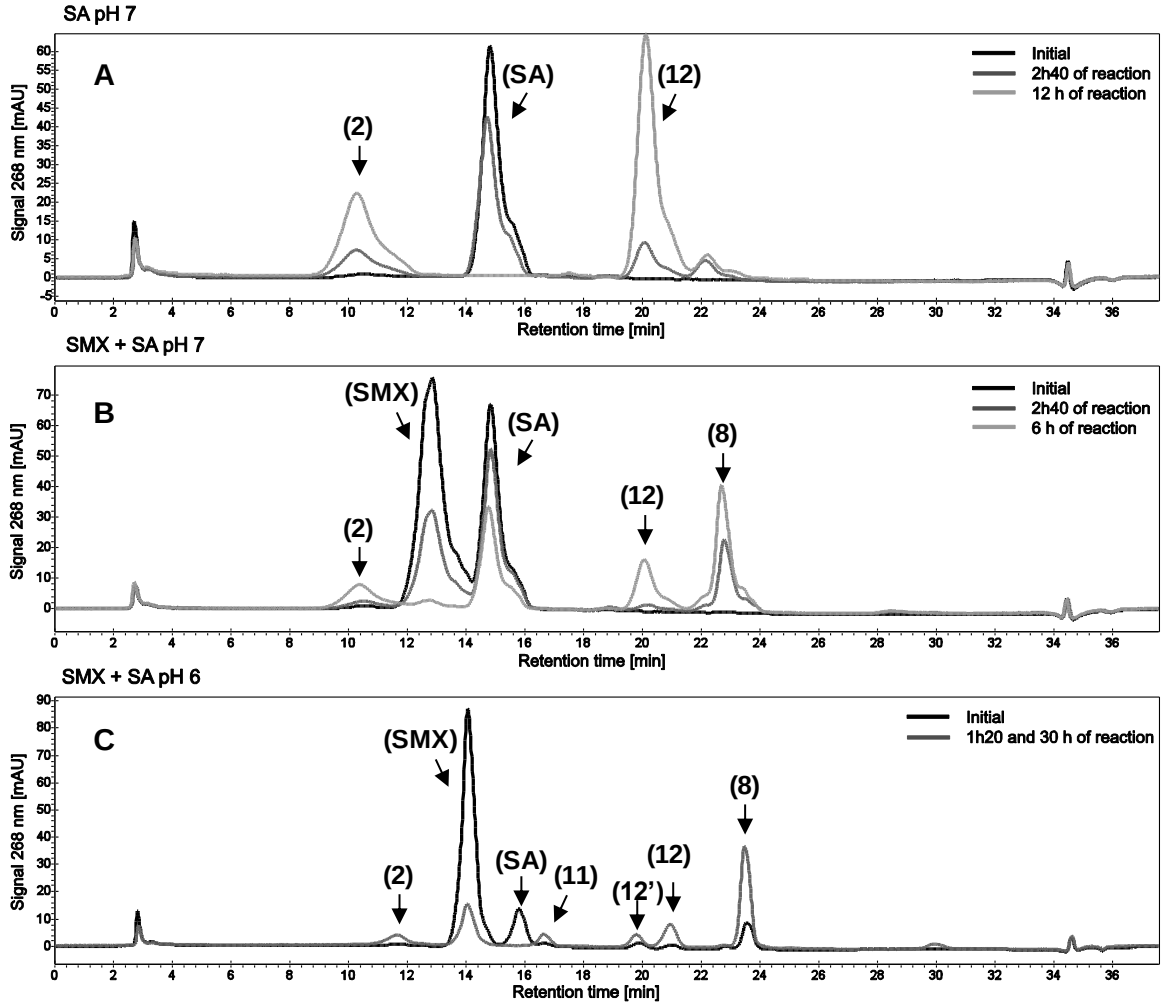

Fig. S 14 HPLC-DAD (268 nm) chromatograms during the oxidation of syringaldehyde (SA) by laccase in presence or absence of sulfamethoxazole (SMX). (A) Oxidation of SA (500 µM) by laccase (450 U l^{-1}) at pH 7, without SMX. (B) Oxidation of SA (500 µM) by laccase (455 U l^{-1}) at pH 7, in presence of SMX (100 µM). (C) Oxidation of SA (100 µM) by laccase (320 U l^{-1}) at pH 6, in presence of SMX (80 µM). Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 3, main manuscript).

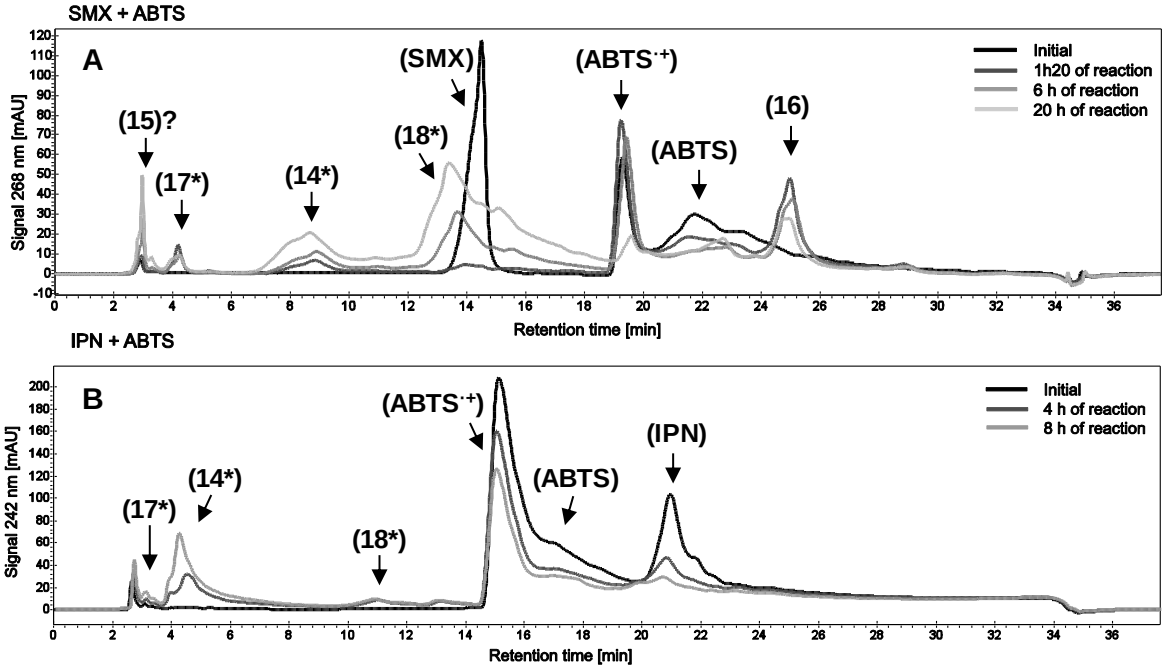

Fig. S 15 HPLC-DAD chromatograms (242-268 nm) during the oxidation of ABTS (500 µM) by laccase in presence of (A) sulfamethoxazole (SMX, 100 µM, pH 6, 450 U l^{-1} laccase, signal at 268 nm), **and in presence of (B) isoproturon** (IPN, 100 µM, pH 5, 503 U l^{-1} laccase, signal at 242 nm). Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 3, main manuscript). Different HPLC methods were used in A and B (peaks not at the same retention time). Transformation products with (*): correspondence between the compounds appearing on the chromatograms by UPLC-MS and HPLC-DAD not completely confirmed.

12 pH influence on the relative abundance of the transformation products

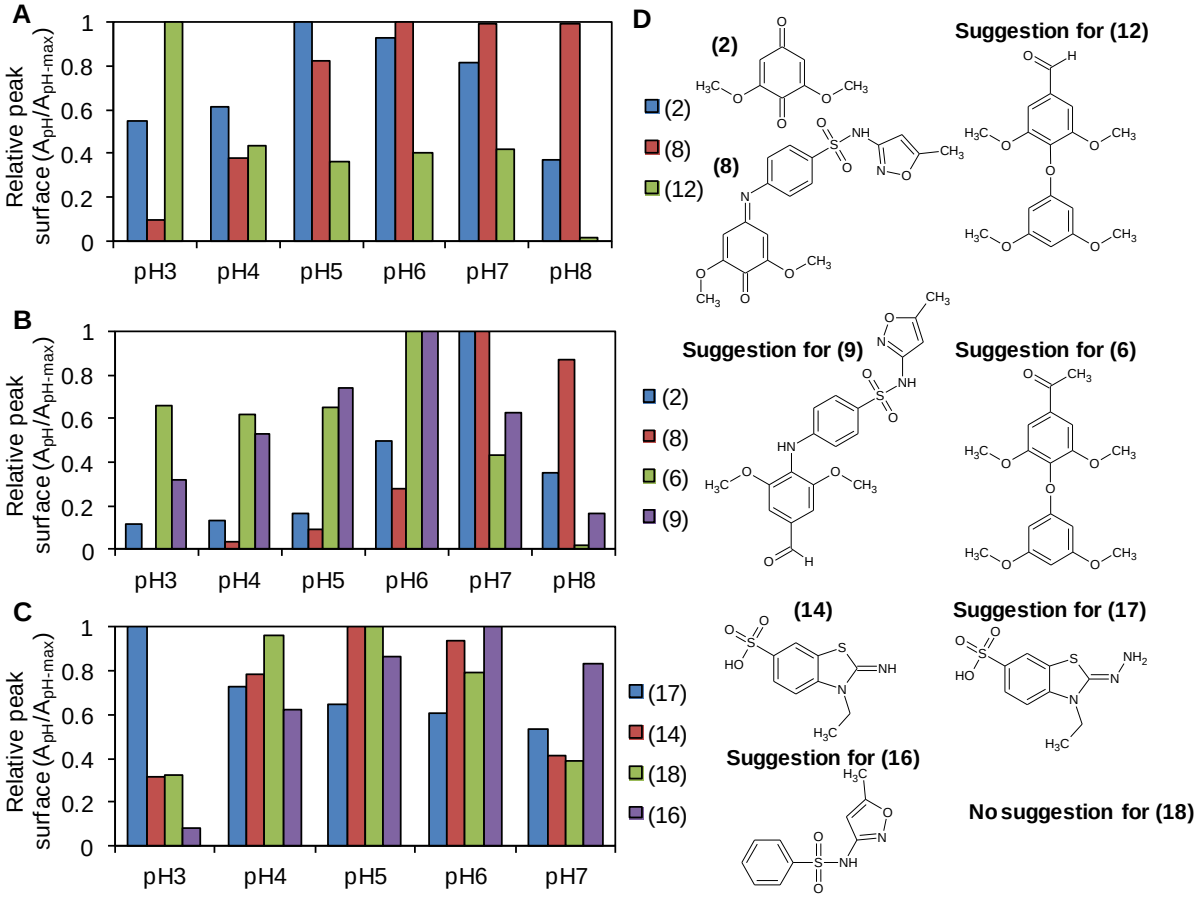

Fig. S 16 Relative abundance of the main transformation products detected by HPLC-DAD after the complete reaction of sulfamethoxazole (SMX) in presence of laccase and mediators at various pH (peak surface of the compound relative to the maximum surface observed for the same compound at different pH). **(A)** In presence of syringaldehyde. **(B)** In presence of acetosyringone. **(C)** In presence of ABTS. **(D)** Structural propositions for the various products. Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 3, main manuscript). For product 9, maximum peak area observed during the reaction (not stable, decrease with the time).

13 Reaction modeling of laccase-mediator systems

Based on the results of this study, especially the kinetics at various pH values and the characterization of the transformation products, a model is proposed to simulate laccase-mediated oxidation processes (Eqs. 1-4). We suggest that the mediator (*med*) is oxidized to a reactive radical ($R^{\cdot}$) in presence of laccase (*lac*) and oxygen (Eq. 1). These radicals can either react by a self-reaction to produce product(s) P_1 (Eq. 2), further react to transformation product(s) P_2 (Eq. 3), or react with the pollutant with a stoichiometric ratio (*a*) (number of moles of reactive radical needed to oxidize one mole of pollutant) to produce product(s) P_3 (Eq. 4). k_1 to k_4 are the respective rate constants of each reaction. This mechanistic description is coherent with the nature of the transformation products detected, as illustrated for SA on Fig. S 17.

$$lac + med + \tfrac{1}{4}O_2 \xrightarrow{k_1} lac + R^{\cdot} + \tfrac{1}{2}H_2O \qquad k_1 \qquad (1)$$

$$R^{\cdot} + R^{\cdot} \xrightarrow{k_2} P_1 \qquad k_2 \qquad (2)$$

$$R^{\cdot} \xrightarrow{k_3} P_2 \qquad k_3 \qquad (3)$$

$$R^{\cdot} + \tfrac{1}{a}\,poll \xrightarrow{k_4} P_3 \qquad k_4 \qquad (4)$$

Analysis of oxygen consumption during the reaction (Fig. S 7) shows that about ¼ mole of oxygen (one mole of electrons transferred) was consumed per mole of mediator (ABTS, AS and SA) oxidized during the first part of the reaction. Oxygen was, however, further consumed during the reaction, probably by additional oxidation of the transformation products (processes not included in the model). In all cases, complete oxygen depletion was observed only after complete pollutant oxidation, suggesting no oxygen limitation in the reaction.

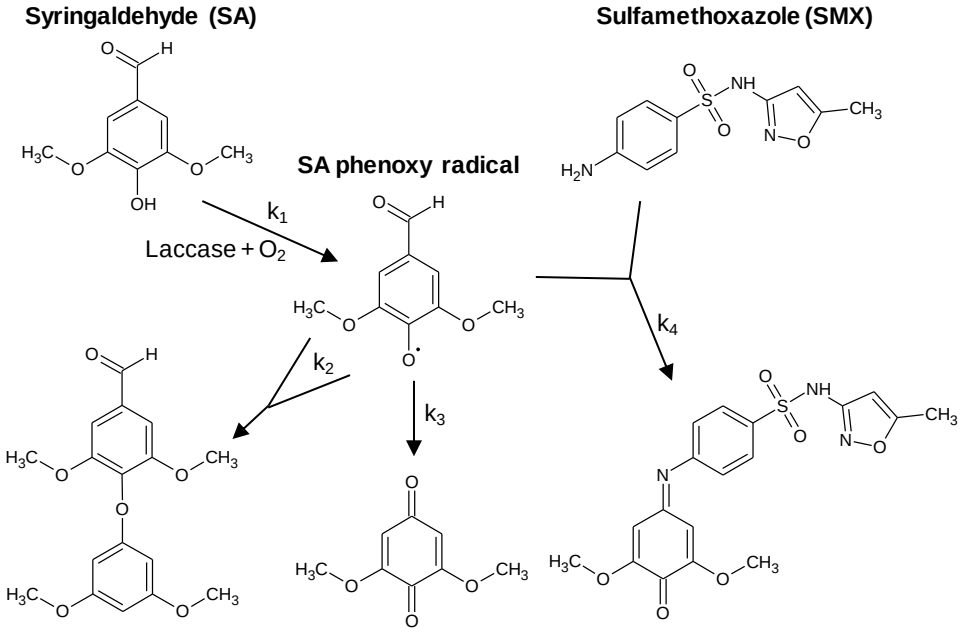

Fig. S 17 Laccase-mediated reactions in solution containing syringaldehyde and sulfamethoxazole, based on the main transformation products observed by UPLC-MS. The structure of the dimeric SA is only hypothesized (not confirmed). k_1 to k_4: rate constants used in the kinetic model.

Based on this reaction model, a kinetic model was established (Eqs. 5-9), assuming a constant laccase recycling (catalytic cycle with no loss of activity during the reaction) (Eq. 5). As the oxidation rate of the mediator by laccase is influenced by the mediator concentration (rate increasing with the concentration until reaching a saturation with a plateau) (Fig. S 18), a Michaelis-Menten type kinetics was used to model the mediator removal rate, with K_m, the specific half-saturation constant of the laccase for a mediator (Eq. 6).

$$\frac{\mathrm{d}[lac]}{\mathrm{d}t} = 0 \tag{5}$$

$$\frac{\mathrm{d}[med]}{\mathrm{d}t} = -k_1[lac][\mathrm{O}_2]\frac{[med]}{K_m + [med]} \tag{6}$$

$$\frac{\mathrm{d}[\mathrm{O}_2]}{\mathrm{d}t} = -\tfrac{1}{4}k_1[lac][\mathrm{O}_2]\frac{[med]}{K_m + [med]} \tag{7}$$

$$\frac{\mathrm{d}[R^{\cdot}]}{\mathrm{d}t} = k_1[lac][\mathrm{O}_2]\frac{[med]}{K_m + [med]} - k_2[R^{\cdot}]^2 - k_3[R^{\cdot}] - k_4[R^{\cdot}][poll] \tag{8}$$

$$\frac{\mathrm{d}[poll]}{\mathrm{d}t} = -\tfrac{1}{a}k_4[R^{\cdot}][poll] \tag{9}$$

This model was used to simulate the behavior of the laccase-mediated reactions under various conditions by assigning arbitrary values (based on rough fitting of the data) to the six undetermined variables k_1, k_2, k_3, k_4, K_m and a. The five differential equations were solved numerically with the ode45 solver (variable step Runge-Kutta method) within Matlab. The values of the parameters used for the simulations shown in the main manuscript (Figs. 5 and 6) are presented in Table S 2.

As presented in the main manuscript, this model was able to qualitatively reproduce all types of experimental results, confirming that the mechanistic description can approximate the laccase-mediated reactions.

Table S 2 Modeling parameters (arbitrary values) used to simulate the reactions presented in Figs. 5 and 6 (main manuscript).

Parameters		**Fig. 5 A**	**Fig. 5 B**	**Fig. 5 C**	**Fig. 5 D**	**Fig. 6**
k_1	[μM^{-1} h^{-1}]	0.1 or 0.001	0.1 or 0.005	0 to 1	0.1	0.1
k_2	[μM^{-1} h^{-1}]	0.005	0.0005	0.005	0.0005	0.005
k_3	[h^{-1}]	0.005	0.0005	0.005	0.0005	0.005
k_4	[μM^{-1} h^{-1}]	0.05	0.005	0.001	0.0015	0.05
K_m	[µM]	10	10	10	10	10
a	[-]	1.7	2.2	1	2.2	1.7
Initial conditions						
O_2	[µM]	250	250	250	250	250
Laccase	[µM]	200	40-200	200	200	200
Pollutant	[µM]	100	100	110	2-200	0.1-150
Mediator	[µM]	10-500	100-500	100	10-1000	0.5-750

14 Determination of the Michaelis-Menten constant K_m

The half-saturation constant K_m for the oxidation of ABTS by commercial laccase from *Trametes versicolor* was determined at pH 4.5 at various ABTS concentrations (1-1000 µM) using a Lineweaver-Burk plot. As shown in Fig. S 18, ABTS oxidation followed Michaelis-Menten kinetics, with a constant K_m value of 19 µM. Similar values (13-25 µM) were reported in other studies for other *Trametes versicolor* laccases [3].

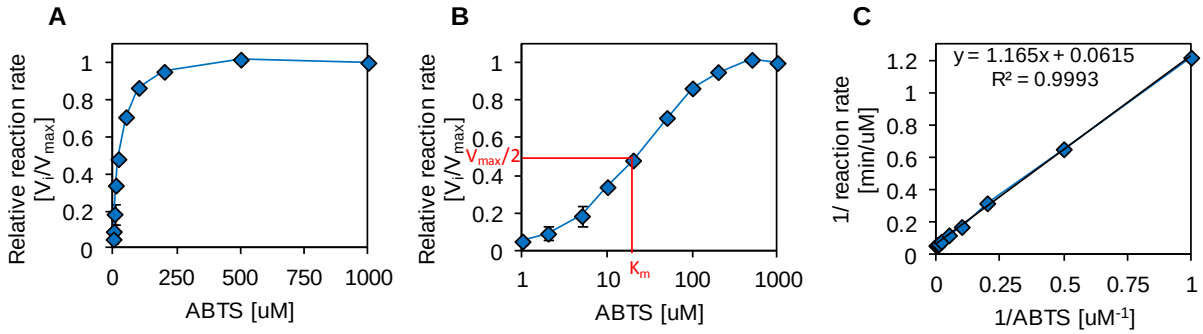

Fig. S 18 Influence of the initial ABTS concentration on the initial ABTS oxidation rate (V_i) by laccase. (A) Linear x-axis scale. (B) Log x-axis scale. (C) Lineweaver-Burk plot of $1/V_i$ as a function of 1/ABTS (linearization of the Michaelis-Menten equation: $(1/V_i) = (K_m/V_{max})(1/[ABTS]) + (1/V_{max})$). V_{max}: maximum rate achieved at saturating substrate concentrations. K_m: substrate concentration at which the reaction rate is half of V_{max}. Conditions: pH 4.5, 25°C, 10 mg l^{-1} comercial laccase from *Trametes versicolor*. The K_m value for ABTS with the selected laccase was found to be 19 µM.

15 References

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